Synthesis of Ectomycorrhizae on Loblolly Pine Seedlings with Basidiospores of Pisolithus tinctorius

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Abstract. Basidiospores of *Pisolithus tinctorius* (2.2 or 0.22 $\times$ 10^6) mixed dry in 800 cm^3 of soil were more effective mycorrhizal inoculum on loblolly pine seedlings than the same quantities of spores suspended in water and added to soil. Roots of nonmycorrhizal seedlings dusted with spores formed more ectomycorrhizae than did seedlings dipped in a water slurry of spores. Water extraction of a spore pigment appeared to decrease spore viability. A concentration of 5.5×10^7 spores mixed in soil prior to planting seed formed significantly more ectomycorrhizae than did greater or lesser spore concentrations. Dry storage in darkness at 5°C from 1 wk to 34 mo of different basidiospore collections did not significantly affect ectomycorrhizal development. Vermiculite and kaolin functioned as physical carriers of basidiospores for soil infestation more effectively than did sand or water. Ectomycorrhizal development from spores was first detected 2 mo after seed germination. A 50 percent increase in ectomycorrhizal development between the fourth and fifth months was associated with a 170 percent increase in seedling growth. These results suggest that basidiospores of *P. tinctorius* can be effectively used in tree nurseries as inoculum to ensure ectomycorrhizal development on tree seedlings. ***Forest Sci. 22:13–20.***

Additional key words. Reclamation of adverse sites, afforestation, reforestation, tree seedling production, *Pinus taeda.*

BASIDIOSPORES are the primary agents for disseminating basidiomycetous ectomycorrhizal fungi. Several workers used basidiospores of specific fungi as inoculum for synthesis of ectomycorrhizae on pine (Marx and Ross 1970) and *Eucalyptus* (Thapar and others 1967). Theodorou (1971) demonstrated that coating of seeds of *Pinus radiata* D. Don with freshly harvested basidiospores of *Rhizopogon luteolus* Fr. and Nordh. prior to planting was an easy and effective way to introduce mycorrhizal fungi into fumigated and nonfumigated soils. Recently, Theodorou and Bowen (1973) reported that after freeze drying and storage for 3 mo at 22°C basidiospores of *R. luteolus* could be used to coat seed. They obtained maximum ectomycorrhizal development on *P. radiata* with 3×10^4 spores per seed. A dose-response curve was also obtained for mycorrhizal infection when basidiospores were applied to soil. At least 10^5 spores in soil per 290 cm^3 pot were needed for maximum ectomycorrhizal development, although mycorrhizae formed on seedlings growing in pots with as few as 100 spores. However, it was necessary to increase the inoculum by 10 times with spores air-dried for 2 days and nearly 100 times for freeze-dried spores for mycorrhizal development comparable to those formed by fresh spores.

Lamb and Richards (1974 a and b) found that basidiospores of *Rhizopogon roseolus* (Corda) T.M. Fr., *Suillus granulatus* (L. ex. Fr.) O. Kuntze, and *Pisolithus tinctorius* (Pers.) Coker and Couch were

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superior to chlamydospores of other fungi as inoculum for mycorrhizal development and growth of *Pinus radiata* in natural soil deficient in fungal symbionts. They found that an increase in inoculum density of viable propagules (i.e., 10^3, 10^6, 10^9 spores per plant) in soil increased the quantity of mycorrhizae. Superphosphate added to a P-deficient soil with low inoculum density also encouraged greater development of ectomycorrhizae.

In recent years, interest has developed in the United States to tailor tree seedlings with specific mycorrhizal fungi for outplanting. This tailoring with fungal symbionts ecologically adapted to the site where seedlings are to be planted could significantly improve survival and early growth of tree seedlings. *Pisolithus tinctorius* is an excellent example of an ectomycorrhizal fungus which is ecologically adapted to adverse sites. Recently, Marx and Bryan (1975) demonstrated that either mycelium grown in vermiculite-peat moss-nutrient medium or basidiospores of *P. tinctorius* could be used to form ectomycorrhizae and stimulate growth of loblolly pine (*Pinus taeda* L.) seedlings in microplots.

Using *P. tinctorius* to improve seedling quality in the nursery and to produce tailored seedlings has excellent potential. However, to use any fungus on a large scale, certain basic criteria must be considered. The availability of viable inoculum in sufficient quantities to infest nursery soils is the first criterion. *Pisolithus tinctorius* produces large basidiocarps that contain many basidiospores, and is distributed throughout the United States and other parts of the world (Mikola 1969). Marx and Bryan (1975) collected over 1300 g of basidiospores of this fungus from basidiocarps under pines in less than 3 hr on a strip-mined coal spoil in northwest Alabama. One g contained approximately 1.1 billion basidiospores. Unfortunately, viability of spores has been difficult to determine (Lamb and Richards 1974c). It appears that the best way to determine basidiospore viability is in actual mycorrhizal synthesis tests.

The purpose of this research was to (i) test different methods of infesting soil and inoculating seedlings with spores; (ii) establish the spore density in soil necessary for ectomycorrhizal development; (iii) evaluate various geographic sources and the effect of storage on spore viability; (iv) determine the efficiencies of different physical carriers used for dispersing spores in soil; and (v) characterize mycorrhizal development on loblolly pine seedlings at monthly intervals.

Materials and Methods

Ectomycorrhizal synthesis tests were performed in an electronically air-filtered, air-conditioned plant growth room. Seedlings were grown in 800 cm^3 of soil mixture in one liter plastic pots (9.5 × 9.5 × 12 cm tall) with drain holes covered with a layer of steamed pebbles. The soil mixture consisted of forest clay loam:sand:peat moss (2:1:1 by volume) steamed for 6 hr at 80° to 85°C on each of 3 alternate days. The loblolly pine seeds were from 1966 and 1972 mixed lots (Eastern Tree Seed Laboratory, USFS, Macon, Georgia). Seeds were presoaked in 1 percent H_2O_2 for 48 hr at 5°C in darkness, and surface sterilized for 30 min in 30 percent H_2O_2. After germination, seedlings were thinned to two per pot and 50 ml of the inorganic salt component of Melin-Norkrans (MMN) nutrient (Marx 1969) were added to each pot.

Basidiospores were removed from basidiocarps of *P. tinctorius* by screening through a 0.84 mesh screen (Marx and Bryan 1975). Only dry, mature basidiocarps (5 to 15 cm diam) with obvious cracks in the peridium and free of insect damage were used. Basidiocarps in advanced stages of peridium deterioration were not collected. In most instances, only the first two or three layers of peridioles nearest the peridium contained sufficiently mature (powder dry) basidiospores for collection and storage. No attempts were made to collect spores from the moist, inner peridiole layers. The spores were stored in plastic bags in amber bottles at 5°C in darkness. At least three samples of basidiospores from each collection were counted with a

hemacytometer. One mg contained 1.1 million spores (± 10 percent). Strands of desiccated, twisted hyphae, 15 to 40μ in length, were present in all spore collections; it was assumed that they were not viable.

Basidiocarps of *P. tinctorius* were collected from under 3-year-old Virginia (*P. virginiana* Mill.) and loblolly pines on a strip-mined coal spoil near Fabius, Alabama, October 1972; 4-year-old loblolly pine on a strip-mined coal spoil near Berea, Kentucky, August 1970; 5-year-old loblolly pine on a strip-mined koalin spoil near Gordon, Georgia, October 1972; 27-year-old shortleaf (*P. echinata* Mill.) pine on a fertile clay site near Raleigh, North Carolina, June 1970; 20 to 25-year-old shortleaf pine on an eroded, Cecil clay soil near Athens, Georgia, September 1969 and October 1972; and from around microplots (Marx and Bryan 1975) of loblolly pine near Athens, Georgia, September 1972.

In initial tests, the powder-dry basidiospores were difficult to wet and keep in water suspension. Even minimal handling caused the spores to be airborne, leading to contamination of pine seedlings in unrelated experiments. To remedy this problem, spores were handled under a hood in the laboratory. After soil infestation, pots of soil were tightly covered to avoid contamination and transported to the growth room where seeds or seedlings were planted.

Seedlings were harvested after 1 to 5 months. Measurements of the two seedlings per pot were averaged as one replicate and all treatments were replicated five times. Foliar-stem fresh weights were recorded and the degree of ectomycorrhizal development was determined by counting mycorrhizal and nonmycorrhizal short roots and calculating percent ectomycorrhizae. Growth stimulation by ectomycorrhizae was determined by comparing foliar-stem fresh weights to those of nonmycorrhizal control seedlings. Analyses of variance were made on all data and means were compared with Duncan's multiple range test at the 95-percent confidence limit. In each test, samples of ectomycorrhizae were surface sterilized in $HgCl_2$ and plated on MMN agar medium for reisolation of *P. tinctorius* (Marx and others 1970).

Soil infestation and direct inoculation of roots. Basidiospores (26 g) for the seven treatments used in this test came from one basidiocarp collected under shortleaf pine near Athens and stored 4 months. Nonmycorrhizal loblolly pine seedlings grown in flats of steamed soil for 3 mo were either transplanted into soil containing basidiospores or inoculated directly with basidiospores prior to transplanting into pots. Soil was infested by mixing 2.2×10^6 spores (100 mg) or 0.22×10^6 spores (10 mg) with 800 cm^3 steamed soil in plastic bags. Spore suspensions were prepared by suspending 2.2×10^6 spores or 0.22×10^6 spores in 10 ml sterile, distilled water with 0.004 percent Tween 20 surfactant, shaking vigorously, filtering through a 53μ stainless steel screen, resuspending in 100 cm^3 sterile, distilled water, and centrifuging at low speed for 15 min to separate spores from surfactant. Concentrated spores were resuspended in 30 ml H_2O, mixed with soil, and nonmycorrhizal seedlings were planted. In two treatments, suspensions of 2.2×10^9 spores (2.0 g) or 5.5×10^8 spores (0.5 g) in 50 ml water were prepared as above and resuspended in 250 ml H_2O. Roots of nonmycorrhizal seedlings were dipped for 5 sec in these spore suspensions before planting. In the last treatment, dry spores were placed on moist roots of nonmycorrhizal seedlings. Prior to planting, the roots of each seedling were inserted into a plastic bag containing 2.2×10^9 spores and the bag shaken to coat the roots with spores. Controls were nonmycorrhizal seedlings planted in steamed soil without spores.

Spore densities in soil. Basidiospores used in this test were from several basidiocarps collected in Alabama and stored for one week. Basidiospores were placed in 7×2 cm glass vials with caps in quantities of 1.0, 0.5, 0.1, 0.05, 0.01, 0.005, and 0.001 g. Controls were 0.1 g basidiospores killed by placing cotton saturated with propylene

TABLE 1. Growth and ectomycorrhizal development of initially nonmycorrhizal 3-mo-old loblolly pine seedlings 2 mo after inoculation with basidiospores of Pisolithus tinctorius.[1]

Number of spores and inoculation method	Ectomycorrhizae[2] (percent)	Seedling growth[3] (percent of controls)
2.2×10^6, mixed dry in soil	12 a	19 a
0.22×10^6, mixed dry in soil	29 b	24 a
2.2×10^6, H_2O suspension mixed in soil	6 a	10 a
0.22×10^6, H_2O suspension mixed in soil	8 a	10 a
2.2×10^6, H_2O suspension, seedlings dipped	18 b	17 a
5.5×10^5, H_2O suspension, seedlings dipped	31 b	15 a
2.2×10^6, dusted onto seedling roots	68 c	88 b
Mean	23.4	26.1

[1] Each value is the mean of five replicate pots per treatment. Means sharing a common letter in each column are not significantly different ($P = 0.05$).

[2] Number of ectomycorrhizae as percentage of total feeder roots.

[3] Percent increase in foliar-stem fresh weights of mycorrhizal seedlings in comparison to nonmycorrhizal control seedlings growing in soil without spores.

oxide in each of the vials containing the spores. After 3 days, the cotton was removed and the vials vented for 5 days before use. Killed spores were used as controls to determine the presence or absence of stimulators to seedling growth to obtain more accurate growth comparisons between seedlings with and without spore inoculum. Spores in vials were suspended in 15 ml of water-surfactant, shaken vigorously, and decanted immediately onto soil in pots. The vials were rinsed twice with additional water and decanted. Spores were mixed into the soil with a spatula to a depth of 5 cm and the pots planted with seed.

Spore source and storage effects. Thirteen basidiospore collections were used in this study. Some were from individual basidiocarps and some were mixtures from several basidiocarps. Duration of spore storage was from 1 wk to 34 mo. Basidiospores (0.1 g) from the various collections were placed in vials and processed as in the spore density study. Control seedlings were grown in soil without spores.

Spores mixed with different physical carriers. Basidiospores of several basidiocarps collected from koalin spoils were used after 6 wk storage. Two particle sizes of commercial vermiculite (grades 2 and 4), koalin (99 percent pure), and white sand were tested as physical carriers for basidiospores. Fifty cm^3 of each carrier were placed in each of five plastic bags. An excess of sterile, distilled water was added, mixed thoroughly, and free water decanted. Either 1.1×10^8 (0.1 g) or 1.1×10^7 (0.01 g) basidiospores were added to each bag and mixed thoroughly. This inoculum was mixed with a spatula into the upper 7 to 8 cm of soil. A water suspension of spores, at both densities, was also mixed with soil for comparison. All pots were planted with seed. Control seedlings were grown in soil without carrier or spores.

Mycorrhizal development on seedlings of different ages. A composite sample of basidiospores from several basidiocarps collected near Athens, Georgia, was used in this test after 3 mo storage. Fifty cm^3 of grade 2 vermiculite were moistened with sterile, distilled water, mixed thoroughly, and free water decanted. Basidiospores (1.1×10^8) were added to and thoroughly mixed in each of 25 bags with vermiculite carrier, incorporated by spatula into the upper 7 to 8 cm of soil in 25 pots, and planted with seed. Fifty cm^3 of vermiculite

TABLE 2. Growth and ectomycorrhizal development of 5-mo-old loblolly pine seedlings in soil containing different quantities of basidiospores of Pisolithus tinctorius.[1]

Basidiospores per 800 cm³ soil		Ectomycorrhizae[2] (percent)	Seedling growth[3] (percent of controls)
Weight (gm)	Number		
1.0	1.1×10^9	26	48
0.5	5.5×10^8	30	58
0.1	1.1×10^8	33	54
0.05	5.5×10^7	48*	83*
0.01	1.1×10^7	32	50
0.005	5.5×10^6	28	52
0.0001	1.1×10^6	27	45
Mean		32	56

[1] Each value is the mean of five replicate pots per treatment; * denotes significance at P = 0.05 confidence level.

[2] Number of ectomycorrhizae as percentage of total feeder roots.

[3] Percent increase in foliar-stem fresh weights of mycorrhizal seedlings in comparison to nonmycorrhizal control seedlings from soil containing 1.1×10^8 spores killed with propylene oxide.

without spores were added to each of 25 pots prior to planting for controls. Seedlings in 5 infested and 5 control pots were harvested each month for 5 mo following seed germination.

Results

P. tinctorius was reisolated from representative ectomycorrhizae in all tests. Ectomycorrhizae were cinnamon brown in color and bifurcate to complex coralloid in morphology. Control seedlings remained nonmycorrhizal.

Soil infestation and direct inoculation of roots. Basidiospores mixed dry in soil synthesized significantly more ectomycorrhizae after 2 mo on initially 3-month-old nonmycorrhizal pine seedlings than did spores suspended in water (Table 1). With dry spores, a concentration of 0.22×10^6 was more effective than the 10-fold greater concentration. This concentration effect was not evident in spore treatments processed in water or those used as a slurry for root dips. Spores dusted onto roots formed more ectomycorrhizae that were more evenly distributed on the roots than spores processed by any other method. Seedlings receiving this spore treatment also grew significantly better than other seedlings.

Spore density in soil. Seedlings with 5.5×10^7 spores per 800 cm³ soil synthesized significantly more ectomycorrhizae than those in soil with greater or lesser spore concentrations (Table 2). Mycorrhizae were not well distributed over the root systems in this test, but were formed in a 3 to 4 cm zone about 1 cm below the root collar. There was a positive correlation between degree of ectomycorrhizal development and percent increase in foliar-stem fresh weights of mycorrhizal seedlings over seedlings without mycorrhizae. Control seedlings with basidiospores killed by propylene oxide were similar in size to seedlings grown without spores in earlier tests.

Spore source and storage effects. Spores collected from different localities and soil conditions and stored dry for as long as 34 mo at 5°C were similar in their capacity to synthesize ectomycorrhizae on loblolly pine seedlings (Table 3). Development of ectomycorrhizae varied as much within as between treatments. Distribution of mycorrhizae on seedlings in this test was similar to that observed in the spore density study.

Spores mixed with physical carriers. Spores mixed with either vermiculite or kaolin formed significantly more ectomycorrhizae than spores mixed with sand; the latter formed more ectomycorrhizae than spores in water carrier (Table 4). There was no significant effect of 10-fold differences in spore density on mycorrhizal development, regardless of physical carrier. Mycorrhizae formed by spores mixed with the vermiculite carrier were more evenly distributed over roots than mycorrhizae formed in the other treatments. As in other tests, growth stimulation was directly cor-

TABLE 3. Growth and ectomycorrhizal development of 5-mo-old loblolly pine seedlings from soil containing 1.1 × 10^8 basidiospores of Pisolithus tinctorius *collected from different basidiocarps and locations from under pine and stored for different lengths of time.*[1]

Location of basidiocarp collection	Time in storage[2]	Basidiocarps (number)	Ectomycorrhizae[3] (percent)	Seedling growth[4] (percent of controls)
Alabama—coal spoils	1 wk	1	50	82
	1 wk	1	31	64
	1 wk	mixed[5]	52	78
	1 wk	mixed[5]	43	58
Georgia—kaolin spoils	3 wk	1	42	74
	3 wk	1	66	87
	3 wk	mixed[5]	53	71
	3 wk	mixed[5]	58	69
Georgia—eroded clay soil	1 wk	1	60	74
	34 mo	mixed[5]	42	63
Georgia—microplot tests	1 mo	mixed[5]	57	60
Kentucky—coal spoils	23 mo	mixed[5]	67	83
North Carolina—clay soil	24 mo	1	41	68
Mean			51	72

[1] Each value is the mean of five replicate pots per treatment. No means were significantly different.
[2] Dry spores stored in clear plastic bags in amber bottles at 5°C in darkness.
[3] Number of ectomycorrhizae as percentage of total feeder roots.
[4] Percent increase in foliar-stem fresh weights of mycorrhizal seedlings in comparison to nonmycorrhizal control seedlings.
[5] Basidiospores from several basidiocarps combined into one collection.

related with the degree of ectomycorrhizal development.

Mycorrhizal development on seedlings of different ages. Ectomycorrhizae were first observed after 2 mo of seedling growth (11 wk from planting) on 2 of 5 seedling pairs (Table 5). These ectomycorrhizae were bifurcate with a minimum of extramatrical growth of hyphal strands into the soil. Significantly more ectomycorrhizae were synthesized and more extramatrical growth of mycelium was observed on roots harvested later. Nearly half of all feeder roots were ectomycorrhizal after 5 mo, and mycorrhizae were evenly distributed over the root systems. Differences in seedling growth were not apparent until after the fourth month. Between the fourth and fifth months there was a 170 percent increase in seedling growth due to approximately a 50 percent increase in ectomycorrhizal development.

Discussion

Basidiospores of *Pisolithus tinctorius* functioned effectively as inoculum for mycorrhizal synthesis on loblolly pine seedlings under a variety of conditions. The viability of these spores after storage for as long as 34 mo at 5°C without prestorage treatments proves their durability. Spores suspended in water-surfactant were less effective as inoculum than spores maintained dry prior to soil infestation. A brown pigment was extracted from the spores in all treatments involving water suspension. Perhaps this pigment has some importance in spore viability; its extraction from spores by water may account for the reduced ef-

TABLE 4. Growth and ectomycorrhizal development of 5-mo-old loblolly pine seedlings from soil containing different numbers of basidiospores of Pisolithus tinctorius *mixed with various physical carriers.*[1]

Physical carrier	Basidiospores (number)	Ectomycorrhizae[2] (percent)	Seedling growth[3] (percent of controls)
Vermiculite, grade 4	1.1×10^8	47 a	63 a
	1.1×10^7	58 a	70 a
Vermiculite, grade 2	1.1×10^8	51 a	64 a
	1.1×10^7	63 a	69 a
Kaolin	1.1×10^8	48 a	51 a
	1.1×10^7	42 a	47 b
Sand	1.1×10^8	31 b	30 b
	1.1×10^7	26 b	32 b
Water	1.1×10^8	17 c	23 c
	1.1×10^7	18 c	20 c
Mean		40	47

[1] Each value is the mean of five replicate pots per treatment. Means sharing a common letter in each column are not significantly different ($P = 0.05$).

[2] Number of ectomycorrhizae as percentage of total feeder roots.

[3] Percent increase in foliar-stem fresh weights of mycorrhizal seedlings in comparison to nonmycorrhizal control seedlings.

fectiveness of water-suspended spores as inoculum. In tests in which the pigment in water was not separated from the spores, such as in the spore density study, the spores appeared to function much more effectively. The surfactant was suspected as inhibiting spore viability, but tests not described here in which spores in water with and without surfactant were compared indicated no differences attributable to the surfactant. Differences in mycorrhizal development between treatments could also be due to different numbers of spores in actual contact with roots. Undoubtedly, dusting spores on roots places more spores in contact with roots than most other methods.

Mixing dry basidiospores with a moist carrier, such as vermiculite, was the most effective method of soil infestation. This eliminated the problem with the pigment extraction and also eliminated accidental contamination of other seedlings by airborne basidiospores. The vermiculite carrier dispersed the spores in soil more evenly than kaolin or sand carriers, dry spores mixed directly with soil, or spores

TABLE 5. Growth and ectomycorrhizal development of loblolly pine seedlings from soil containing 1.1×10^8 basidiospores of Pisolithus tinctorius *in vermiculite carrier assayed at monthly intervals for 5 mo.*[1]

Time from seed germination	Ectomycorrhizae[2] (percent)	Seedling growth[3] (percent of controls)
1 mo	0	3 a
2 mo	'2 a	9 a
3 mo	11 b	13 a
4 mo	31 c	21 b
5 mo	47 d	59 c

[1] Each value is the mean of five replicate pots per treatment. Means sharing a common letter in a column are not significantly different ($P = 0.05$).

[2] Number of ectomycorrhizae as percentage of total feeder roots.

[3] Percent increase in foliar-stem fresh weights of mycorrhizal seedlings in comparison to nonmycorrhizal control seedlings.

[4] Two of five seedling pairs had ectomycorrhizae at this time.

suspended in water and mixed with soil, as shown by the degree of mycorrhizal development and the distribution of mycorrhizae on the root systems. Further, in conventional nursery experiments not reported here, dry basidiospores of *P. tinctorius* mixed with moist vermiculite, broadcast on fumigated soil, and mixed into soil mechanically were also effective mycorrhizal inoculum (D. H. Marx, unpublished data).

Significantly more ectomycorrhizae developed with 5.5×10^7 spores per 800 cm^3 soil than with greater or lesser densities. Why more spores did not form more mycorrhizae is not understood. Perhaps inhibitors are present in basidiospores of *Pisolithus* which at high densities inhibit their germination.

Basidiospores of *P. tinctorius* did not form enough mycorrhizae to stimulate seedling growth until the fourth month. This is somewhat longer than required for mycelial inoculum of the fungus tested under similar conditions (Marx 1973). Under nursery conditions, where inocula of other ectomycorrhizal fungi are present, this delay in formation of mycorrhizae by basidiospores could allow colonization of seedling roots by other fungi as well as *P. tinctorius*. It should be pointed out that none of the findings reported here involved competition between *P. tinctorius* and other symbiotic fungi.

The fact that large volumes of basidiospores of *P. tinctorius* are available in nature and that these spores function as inoculum for mycorrhizal synthesis on pine indicates that *P. tinctorius* has excellent potential for use in pine nursery operations. Additionally, the ability of the basidiospores to survive simple storage following collection is important. *P. tinctorius* normally produces basidiocarps in late summer or early fall. Therefore, spores collected during these periods could easily be stored through the winter and still be effective as mycorrhizal inoculum in nurseries in the spring.

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